



## Fractionation of low-molecular-mass heparin by centrifugal partition chromatography in the ion-exchange displacement mode

Olivier Intes<sup>a</sup>, Jean-Hugues Renault<sup>a,\*</sup>, C. Sinquin<sup>b</sup>, Monique Zèches-Hanrot<sup>a</sup>,  
Jean-Marc Nuzillard<sup>a</sup>

<sup>a</sup>Laboratoire de Pharmacognosie, UPRES-A CNRS 6013, CPCBAI, BP 1039, Moulin de la Housse, F-51097 Reims Cedex, France

<sup>b</sup>URM2, Lab. VP/BM, IFREMER, Rue de l'Île d'Yeu, BP 21105, F-44311 Nantes Cedex 3, France

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### Abstract

Centrifugal partition chromatography in the ion-exchange displacement mode allowed a preparative and efficient fractionation of low-molecular-mass heparins from enoxaparin sodium. Amberlite LA2 — a lipophilic liquid secondary amine — was chosen as a weak anion exchanger. The biphasic system methyl isobutyl ketone–water was selected. Protonated LA2 (10%, v/v) was added to the organic stationary phase. Hydroxide ( $\text{Na}^+$ ,  $\text{OH}^-$ ) was chosen as a displacer in the aqueous mobile phase. The observed pH and concentration profiles are typical of displacement chromatography, as supported by numerical simulation. The Dubois test for the analysis of sugar content and an analysis of sulfur content (and consequently sulfatation rate) were carried out to monitor the effectiveness of the procedure. Moreover, the fractions were analyzed by high-performance size-exclusion chromatography and the  $^1\text{H}$  NMR spectra confirmed the fractionation of the sample of enoxaparin sodium. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Heparin; Carbohydrates; Glycosaminoglycans; Enoxaparin sodium; Centrifugal partition chromatography

### 1. Introduction

Enoxaparin sodium (Lovenox) is the commercial name of the substance obtained by alkaline hydrolysis of heparin benzylic esters. It contains a complex mixture of low-molecular-mass heparins. These molecules are polysulfated polysaccharides. They belong to the family of glycosaminoglycans: a highly sulfated linear sugar chain composed of repeated disaccharidic units of uronic acids and

glycosamine. In this pattern four positions can be sulfated as shown in Fig. 1.

Enoxaparin sodium contains on average eight disaccharide units with a mean molecular mass of 4500 Da (mostly comprised between 2000 and 8000 Da). Its antithrombotic and anticoagulant properties, inherited from heparins, confer on it a fundamental significance in surgery and medicine. Its fractionation and characterization have proved to be difficult. Different techniques have been used to analyze and/or fractionate heparins such as size-exclusion chromatography [1], ion-exchange chromatography on solid support [2], capillary electrophoresis [3], or counter-current chromatography in the elution mode [4,5]. Ion-exchange centrifugal partition chromatog-

\*Corresponding author. Tel.: +33-3-2691-3548; fax: +33-3-2691-3596.

E-mail address: jh.renault@univ-reims.fr (J.-H. Renault).

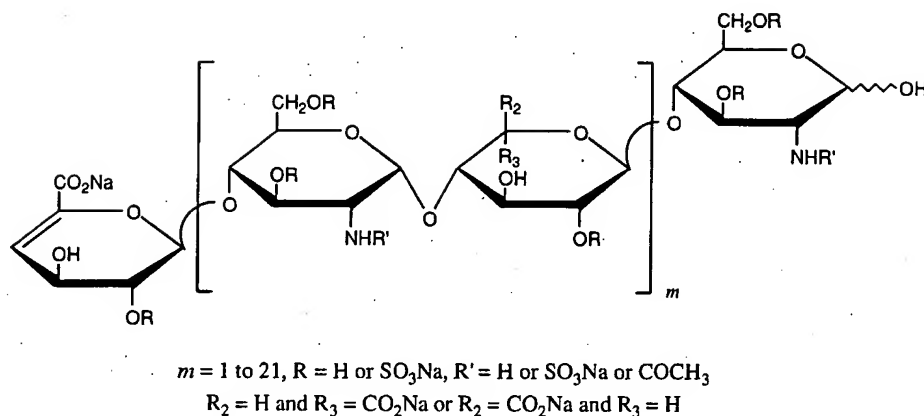


Fig. 1. The structure of heparins.

raphy seemed to be an appropriate technique for such a chromatographic analysis [6].

Centrifugal partition chromatography (CPC) [7] is a non-solid-support, liquid–liquid chromatography, that uses two immiscible solvents (or solutions) prepared from an equilibrated liquid–liquid biphasic system. One phase is kept stationary by a constant centrifugal field whereas the other liquid is pumped through it, thus playing the role of a mobile phase. The analytes are separated according to their partition coefficient.

This type of chromatography is now widely used for preparative separations of fragile biomolecules [7]. Indeed, it avoids problems related to interaction and/or irreversible adsorption of the injected material with a solid support. Moreover, there is generally no problem due to saturation of the stationary phase, allowing CPC to be efficient in preparative separation [8]. Finally, various chromatographic development modes are available, depending on the chemical nature of the sample. In 1994, Ito and Ma [9] successfully applied the displacement mode in CPC and named it pH-zone refining. Displacement was performed through acid–base equilibrium allowing the separation of ionizable compounds, whose electric charge is pH dependent, according to  $pK_a$  and partition coefficients. This chromatographic process forces the target molecules to endure ionization state changes introducing possible degradation of fragile analytes. The solutes also need to present a significant solubility difference between their neutral and charged forms.

As previously mentioned, enoxaparin sodium con-

tains very polar molecules which bear numerous negative charges of the sulfate and carboxylic groups. Heparins are not ionizable but anionic compounds that are always water soluble, consequently pH-zone refining is not effective to fractionate these molecules.

In 1998, Chevolut et al. [6,10] introduced a new type of displacement mode in CPC: ion-exchange. The principle of this method consists of generating lipophilic ion-pairs in the organic stationary phase. Amberlite LA2, a weak anion exchanger is used in order to significantly alter the polarity of the target molecules. LA2 is miscible with almost all common organic solvents and immiscible with an aqueous solution. The displacement process is carried out by injecting a strongly basic mobile phase. The hydroxy ions displace all anions from the stationary phase to the mobile one through a progressive neutralization of the weak anion exchanger. For good separation, the association strength of LA2 with the anionic analytes depends on the nature of the latter. Analytes emerge from the CPC column in characteristic blocks detected as shaped peaks of homogeneous material [6].

The present study shows the use of ion-exchange displacement applied to centrifugal partition chromatography for the fractionation of low-molecular-mass heparin. The starting material is extremely heterogeneous in its chemical nature and consequently the analysis of the collected fractions was an extremely complicated problem. For this preliminary work, the goal was to show that the chemical composition of the different fractions was different from that of the

starting material. Besides pH monitoring, the collected fractions were subjected to different analytical methods: Dubois assay to specify the presence of carbohydrates, high-performance size-exclusion chromatography (HPSEC) analysis and sulfur content evaluation to determine, respectively, the distribution of the relative molecular masses and the evolution of the sulfatation rate. Finally  $^1\text{H}$  NMR spectra of characteristic fractions proved unambiguously the chemical structure differences and thus, the effective fractionation of enoxaparin sodium.

## 2. Experimental

### 2.1. CPC apparatus

The separations were performed using a HPCPC Sanki Series 1000 column (Tokyo, Japan). The column is a stacked circular partition disk rotor which contains 2136 partition cells with a total internal volume of about 240 ml. The column was connected to the injector and the detector through two high-pressure rotary seals. A four-port valve, integrated to the CPC apparatus, allowed it to be operated in either the ascending or the descending mode depending on the relative density of the mobile and stationary phase. The HPCPC was connected to a Techlab (Erkerode, Germany) TIP50 gradient pump. Detection was performed with a microflow pH electrode (Cole-Parmer, Vernon Hills, IL, USA) connected to a pH meter type PHM240 (Radiometer, Copenhagen, Denmark). Fractions were collected with a collector model Superfrac manufactured by Pharmacia (Uppsala, Sweden). Sample injections were carried out by a Rheodyne valve type 7125 (Altech, Deerfield, IL, USA), through a 20-ml loop.

### 2.2. Reagents

Methyl isobutyl ketone (MiBK, puro), 35% hydrochloric acid, 1 M sodium hydroxide solution were purchased from Carlo Erba (Rodano, Italy); Amberlite LA2 from Rohm & Haas (Philadelphia, PA, USA);  $\text{D}_2\text{O}$  from Sigma-Aldrich (St. Louis, MO, USA). A sample of enoxaparin sodium was kindly provided from the Central Pharmacy of the Regional Hospital in Reims.

### 2.3. Preparation of solvent phases and sample solutions

#### 2.3.1. Organic stationary phase

MiBK containing 10% (v/v) of LA2 and water were thoroughly equilibrated in suitable proportions (1:1 v/v) and the two phases were separated. The average molarity of LA2 in the organic phase was 0.225 mequiv.  $\text{ml}^{-1}$ . A sufficient amount of 35% aqueous HCl (19.9 ml for 1 l of organic phase) was added to the upper organic phase to fully protonate LA2.

#### 2.3.2. Aqueous mobile phase

A 0.025 M solution of NaOH was saturated with a 10% solution of unprotonated LA2 in MiBK. A small amount of organic phase was sufficient to fully saturate the aqueous phase, as LA2 and MiBK are practically insoluble in water.

#### 2.3.3. Sample solution

Enoxaparin sodium (2 g) was dissolved into a volume of 20 ml (19 ml of the stationary organic phase and 1 ml of the aqueous mobile phase). The addition of a small amount of the conjugated phase in the injection volume is sometimes necessary to completely dissolve the sample, and to decrease the risk of perturbing column equilibrium.

### 2.4. Separation procedure

The column was first filled with the stationary phase. Then, the rotation speed was set to 1000 rev./min, and the sample was injected. The mobile phase was pumped into the column in descending mode at a flow-rate of 5 ml/min resulting in 30 bar of backpressure. From fraction 23 the flow-rate was set to 2.5 ml/min and the backpressure dropped to 15 bar. The separation was checked by the evolution of the pH, the latter being continuously monitored by an on-flow pH electrode.

### 2.5. Analytical controls

#### 2.5.1. Carbohydrate analysis

The carbohydrate amount in each fraction was evaluated by the phenol-sulfuric acid method of Dubois et al. [11]. The starting material was used as

reference. The absorbance at 482 nm was measured using a UV–visible spectrophotometer type Philips PU 8720 (Bobigny, France).

#### 2.5.2. Sulfur content

The sulfur content of the fractions of interest was measured by the 'Service Central d'Analyse du C.N.R.S.' (Vernaison, France).

#### 2.5.3. Relative molecular mass determination

An aliquot of each fraction was analyzed by HPSEC in 0.2 M NaCl using a 30×0.32 cm I.D. Superdex column (Pharmacia PC 3.2/30) at a flow-rate of 0.1 ml min<sup>-1</sup>. Column calibration was performed with standard pullulans. Pullulans are neutral glucans, whereas heparins are highly negatively charged oligosaccharides. Consequently, such calibration does not allow an exact measurement of enoxaparin molecular mass and was used only for relative comparisons. Area measurements and molecular mass calculations were achieved using the Turbochrom software (Perkin-Elmer, Norwalk, CT, USA).

#### 2.5.4. <sup>1</sup>H NMR analysis

Spectra were recorded at 500 MHz in <sup>2</sup>H<sub>2</sub>O on an Avance DRX 500 Bruker spectrometer (Karlsruhe, Germany).

### 3. Results and discussion

#### 3.1. CPC separation

The selected operating conditions are presented in Table 1. In all cases, a steady 'bleeding' of the organic stationary phase starts at the beginning of the mobile phase emergence. This leakage decreases and finally stops suddenly just before the emergence of sugar-containing fractions. Fig. 2 shows that fraction 3 (before emergence of the mobile phase) and fraction 70 (at emergence of analytes) are strictly monophasic whereas fractions from 8 to 65 contain emulsified phases (fraction 10 and 30 are presented as typical examples). Total 'bleeding' of the stationary phase after the equilibration of the column is 66 ml, corresponding to 32% of the final stationary phase retention. This problem is caused by an emulsion effect arising from both the physico-chemical features of enoxaparin and the ionization state of the anion exchanger. It does not cause any negative effect during the emergence of the analytes on the pH profile or in the separation process since there is no trace of the anion exchanger in the aqueous phase. Moreover, the recovery ratio of the injected material is 92% thus proving that there is no heparin lost due to phase emulsion.

#### 3.2. Sugar test (Dubois test)

The Dubois assay was performed from fraction 9

Table 1  
Experimental conditions of the fractionation of enoxaparin sodium by CPC using Amberlite LA2 as weak anion exchanger

|                               |  |
|-------------------------------|--|
| Apparatus                     | HPCPC Sanki Series 1000 (240 ml)   |
| Biphasic solvent system       | MiBK-LA-2-water (4.5:0.5:5, v/v)   |
| Stationary phase              | Upper organic phase +18.7 ml l <sup>-1</sup> HCl 37%                                       |
| Mobile phase                  | Lower aqueous phase +NaOH 0.025 M  |
| Sample mass                   | 2 g of low-molecular-mass heparins   |
| Injection volume              | 20 ml (19 ml of stationary phase and 1 ml of mobile phase)                                 |
| Rotation speed                | 1000 rev./min  |
| Flow rate                     | 5 ml/min then 2.5 ml/min after 1 h 55 min of pumping                                       |
| Back pressure (bar)           | 37 bar after the column equilibration decreasing to 17 bar when the heparins are collected |
| Retention of stationary phase | 60% after the column equilibration and 32% at the end of the experiment                    |
| Detection                     | Online pH monitoring   |
| Fraction size                 | 4 min then 2 min from fraction 25 to the end   |

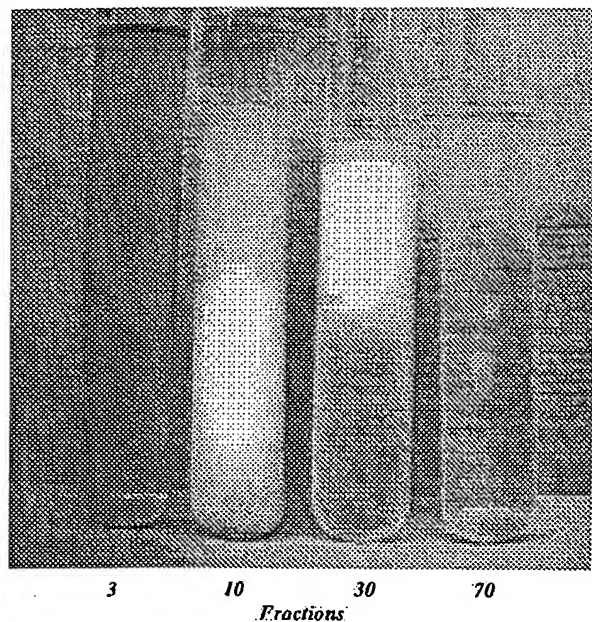


Fig. 2. Visualisation of the stationary phase leakage. Fractions 1 to 9 contain only stationary phase and correspond to the equilibration of the CPC column. During the progression of the injected material into the CPC column, the collected fractions (10 to 67) contain an emulsified mixture of the two phases. The solution becomes clear when the heparins are collected (fraction 68 to 97).

(emergence of the mobile phase) to 92. A significant absorbance at 482 nm is measured from fraction 68 to 87. These absorbance profiles are shown in Fig. 3. The observed rectangular shape is in good agreement with compressive fronts obtained in the displacement chromatographic mode.

### 3.3. Chromatogram (pH profile)

As shown by Chevolot et al. [6], the pH of the mobile phase during the emergence of the hydrophilic compounds (heparins in our case) reflects the stability of the ion pairs  $\text{LA2H}^+, \text{Hep}_i^-$  in organic phase (i.e. the conditional acidity of  $\text{LA2H}^+$  in the presence of heparins  $\text{Hep}_i^-$ ).

The concentration of the chemical species present in the column effluent can be evaluated assuming the following hypotheses on the chromatographic process.

- (i) The column is divided into a limited number of partition cells that mimic chromatographic theoretical plates.
- (ii) Each cell is divided in two parts, of volume  $V_{\text{aq}}$  and  $V_{\text{org}}$ , respectively, corresponding to the aqueous mobile and organic stationary phase. The ratio  $V_{\text{org}}/V_{\text{aq}}$  is noted  $v$ .

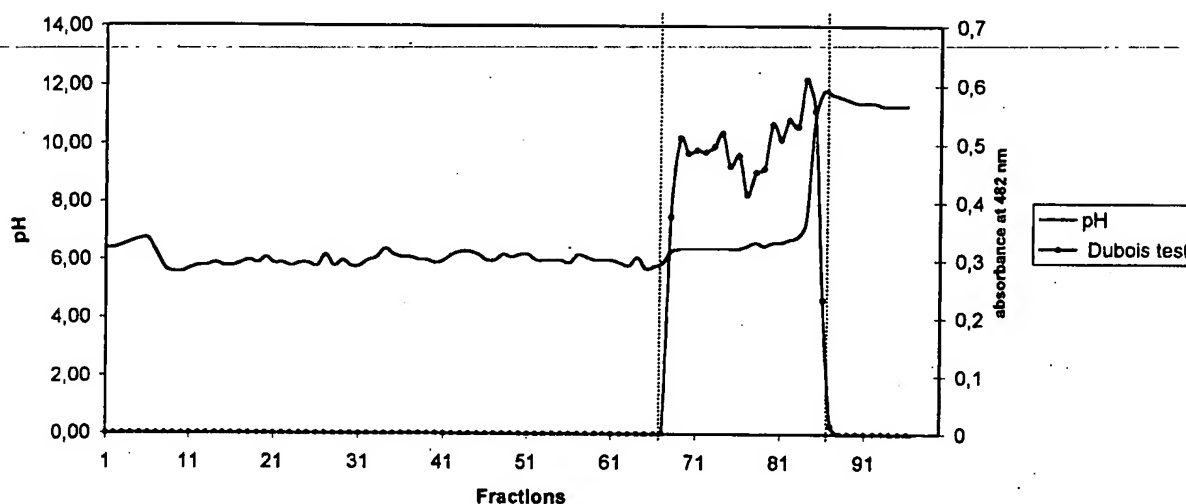


Fig. 3. pH profile and sugar content (Dubois assay) monitoring during the separation process of enoxaparin sodium.

(iii) The pumping of the mobile phase is decomposed into the repetition of two elementary processes: chemical equilibrium between the present species and transfer of the mobile phase content from each cell to the following one. Fresh mobile phase containing the displacer ( $\text{Na}^+$ ,  $\text{OH}^-$ ) is introduced in the first cell while the last one is collected and analysed [12].

(iv) The weak anion exchanger is a highly lipophilic monoamine, it is therefore never present in the aqueous mobile phase. The stationary phase initially contains the ion exchanger Ex (namely LA2) as its hydrochloride  $\text{ExH}^+\text{Cl}^-$ .

(v) The  $n$  analytes are strong acids  $\text{HS}_i$  ( $1 \leq i \leq n$ ), present in the mobile phase as  $\text{S}_i^-$  and in the stationary phase as the ion pair  $\text{ExH}^+\text{S}_i^-$ .

The equilibration step must be carried out, in each cell, each time a volume  $V_{\text{aq}}$  of the mobile phase is pumped into the column. The computation of equilibrium concentrations requires the knowledge of initial concentrations and thermodynamic constants.

In each cell,  $n_0$ ,  $e_0$  and  $s_i$  represent the ratio of the total number of moles of  $\text{Na}^+$ , Ex and  $\text{S}_i^-$  to the volume  $V_{\text{aq}}$ . The overlined symbols are used for concentrations and species in the organic phase.

$$n_0 = [\text{Na}^+] \quad (1)$$

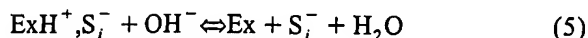
$$e_0 = v([\text{Ex}] + [\text{ExH}^+\text{S}_i^-]) \quad (2)$$

$$s_i = [\text{S}_i^-] + v[\text{ExH}^+\text{S}_i^-] \quad (3)$$

The index  $i=0$  can be assigned to  $\text{Cl}^- = \text{S}_0^-$ . Indeed, Ex is initially saturated with HCl, and thus  $\text{Cl}^-$  ions play the role of retainer. The chemical equilibrium is imposed by the thermodynamic equilibrium constants  $K_i$  ( $0 \leq i \leq n$ ):

$$K_i = \frac{[\text{Ex}][\text{S}_i^-]}{[\text{ExH}^+\text{S}_i^-][\text{OH}^-]} \quad (4)$$

corresponding to the neutralisation reaction of the ion pairs by the displacer:



Taking into account the auto-dissociation of water (Eq. (6)) and the electric charge neutrality of the

system leads to a set of two non-linear equations: Eqs. (7) and (8):

$$K_e = [\text{H}^+][\text{OH}^-] \quad (6)$$

$$e_0 = v\alpha \left( [\text{OH}^-] + \sum_{i=0}^n \frac{s_i}{K_i + v\alpha} \right) \quad (7)$$

$$\frac{K_e}{[\text{OH}^-]} + n_0 = [\text{OH}^-] + \sum_{i=0}^n \frac{s_i K_i}{K_i + v\alpha} \quad (8)$$

The unknowns are  $[\text{OH}^-]$  and  $\alpha = [\text{Ex}]/[\text{OH}^-]$ .

Once the equations are solved, the concentration of the analytes in the two phases in each cell and at any time are obtained considering that:

$$[\text{S}_i^-] = \frac{s_i K_i}{K_i + v\alpha} \quad (9)$$

$$[\text{ExH}^+\text{S}_i^-] = [\text{S}_i^-] \cdot \frac{\alpha}{K_i} \quad (10)$$

The resolution of Eqs. (7) and (8) resorts to the modified Powell method [13] as implemented in the SNSQ algorithm of the CMLIB library [14].

A typical run of the simulation process is presented in Fig. 4, in which the initial concentration of exchanger is 0.2 M, and that of NaOH in the mobile phase is 0.1 M. The number of cells is 40 and the first one is loaded with a solution of  $\text{Na}^+\text{S}_i^-$  ( $i = 1, 2, 3$ ) at a 2 M concentration each. The  $K_i$  values ( $i = 0, 1, 2, 3$ ) are  $10^6$ ,  $10^3$ ,  $5 \times 10^2$ ,  $10^2$ , respectively. An overall 140  $V_{\text{aq}}$  of aqueous phase is pumped, the first 40 are those for which the chromatographic front progresses through the column.

Even though this simulation represents only a rough model of what really happens inside of the column, the general features of displacement chromatography are clearly visible: the analytes flow out of the column as blocks at a constant concentration equal to that of the displacer. This behavior is well known in the field of displacement chromatography using ion exchangers either liquid or solid. The pH value progresses by steps, being constant when an analyte is present at a constant concentration.

The recorded pH profile (Figs. 3 and 5) corresponds to a typical displacement chromatographic run resulting from the neutralisation of different weak acids ( $\text{LA2H}^+$  associated with various anionic species) by a strong base ( $\text{OH}^-$ ). Firstly, the plate

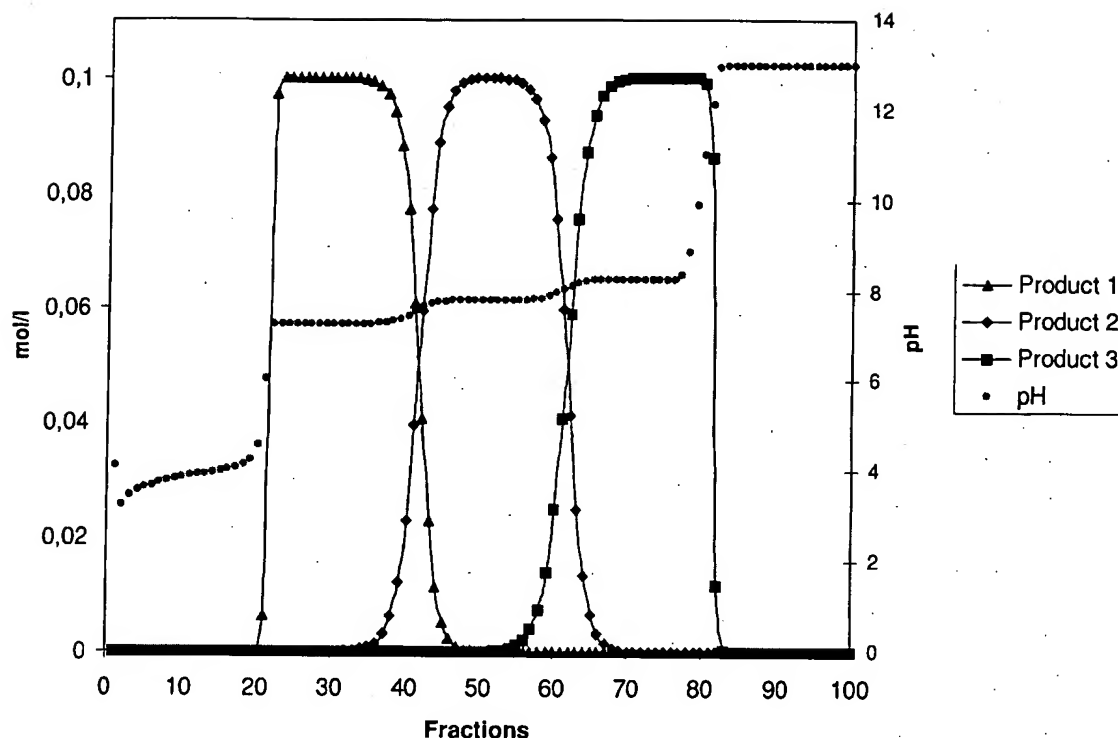


Fig. 4. Simulated pH and concentration profile of a separation of three analytes, according to the model defined in Section 3.3 (see text).

from fraction 9 to 67 (presenting a small variation due to the stationary phase bleeding) corresponds to the neutralisation of the weak acid  $\text{LA2H}^+$  associated with  $\text{Cl}^-$  by aqueous  $\text{OH}^-$  as a mobile phase. Then, the pH profile shows a zone of transition consisting of a series of plateaux (with slight increases of pH values) from fraction 68 to 83 corresponding to the neutralisation of  $\text{LA2H}^+$  associated with various polyanionic species of low-molecular-mass heparins (i.e. corresponding to a continuous displacement of heparin species arranged by homogenous families). A more detailed analysis of this zone reveals four plateaux: the first from fraction 68 to 76, the second from fraction 77 to 79, the third from fraction 80 to 81 and the fourth from 82 to 83.

The pH reflects the stability of  $\text{LA2H}^+$ ,  $\text{Hep}^-$ , a pH plateau corresponds to the elution of a homogeneous mixture of low-molecular-mass heparins in this chromatographic process. Thus, these four plateaux show that the injected sample seems to be fractionated in four mixtures, the chromatographic

behavior of the different species being homogeneous in one zone.

Finally, the pH increases abruptly from fraction 84 to 87 and stabilises permanently to the value of the pH mobile phase indicating the full neutralisation of the anion-exchanger and consequently the end of the chromatographic process.

### 3.3.1. HPSEC analysis

SEC analysis was performed for the Dubois assay positive fractions (68 to 87). Fig. 6 shows typical SEC chromatograms of CPC fractions. It appears that the SEC patterns of fractions 70 and 76 present two partly overlapping peaks whereas the one of fraction 80 shows only one peak. For each fraction, the retention times of the top of the peaks are plotted in Fig. 5. This fractogram shows the evolution of the relative molecular masses during the chromatographic process. It can be seen that two main families of heparins emerge according to the SEC selectivity criteria. The first one divides up in the fractions

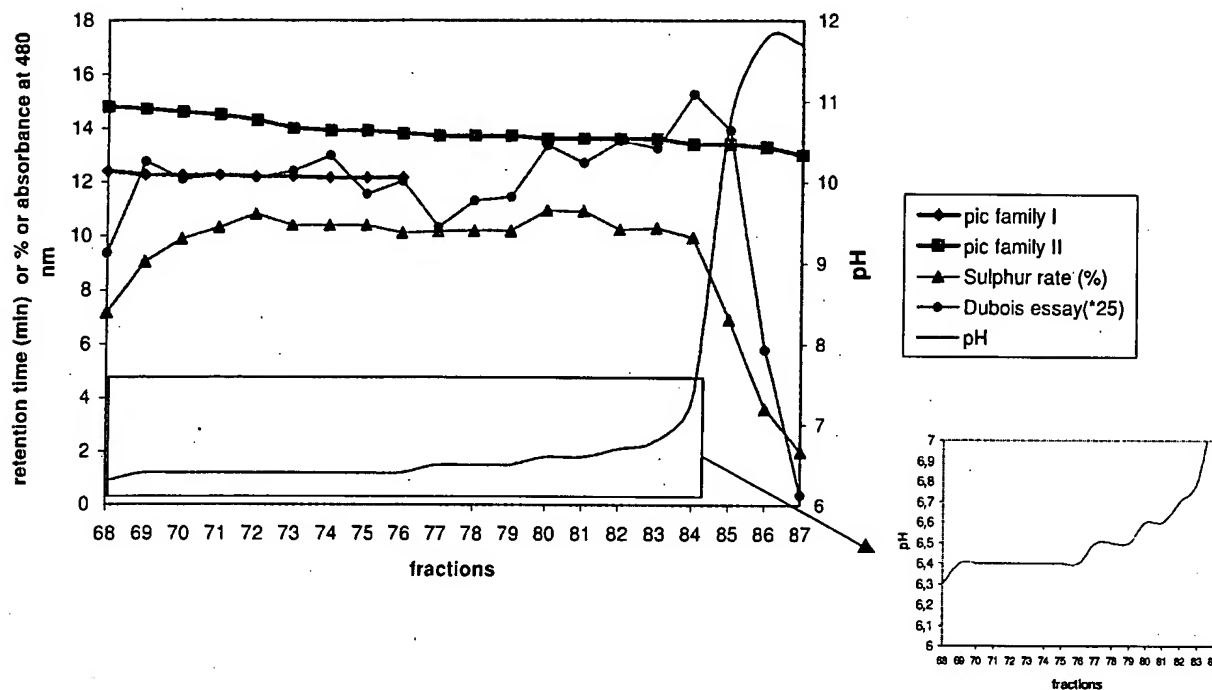


Fig. 5. pH profile, sugar content, sulphur rate and HPSEC analysis of the CPC fractions containing low-molecular-mass heparins.

corresponding to the beginning of the elution of the analytes (from fraction 68 to 76). It is constituted exclusively of the highest molecular masses (giving the lowest retention time during the SEC analysis). For this family, the retention times decrease from 12.4 min for the fraction 68 to 12.15 min for the fraction 76. It indicates that for a family of heparins the molecular masses increase during the emergence

of these compounds. This observation is in agreement with the theoretical model of the chromatographic process: the higher the number of sulfated osidic units, the higher the number of associations with LA2, and the more difficult the displacement of the heparins in the aqueous mobile phase.

The second family is divided in all the Dubois assay positive fractions. On the whole, this corre-

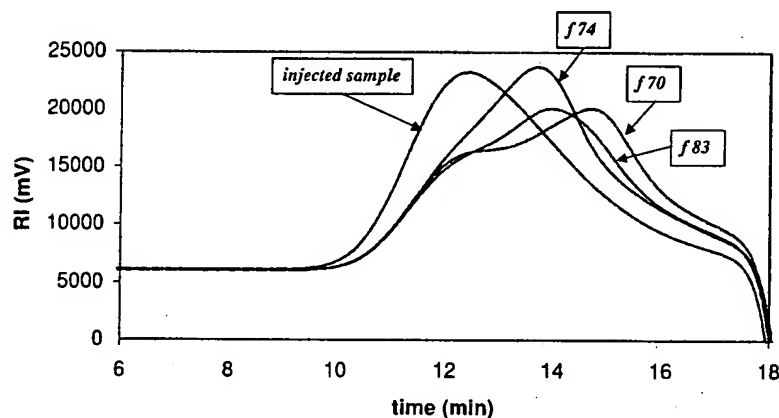


Fig. 6. HPSEC chromatograms of three representative fractions and of the injected sample.



sponds to molecules of lower mass than those in the first family and molecular mass decreases from fraction 68 (14.8 min as retention time) to fraction 88 (13 min as retention time).

Because of the low difference in the molecular masses it seems impossible to separate these molecules using preparative SEC.

### 3.3.2. Sulfur rate analysis

Sulfur content was performed for the Dubois assay positive fractions (68 to 87). Fig. 5 shows the evolution of the sulfur rate during the CPC separation. The sulfur content appears roughly constant but five zones can be defined according to the shape of the curve. The first includes the fractions from 68 to 76, the second from 77 to 79, the third from 80 to 81, the fourth from 82 to 83 and finally the fifth from 84 to 87. The transitions between these different zones are identical to the ones defined during pH profile analysis. This observation was confirmed by the modelisation of the experimental profiles as a constant piecewise function. The location of the transitions between the pieces are chosen so that the residue between model and experimental data is minimal in the least-squares sense.

### 3.3.3. Fractionation evaluation by $^1\text{H}$ NMR

The goal of the  $^1\text{H}$  NMR study was not to hypothesize on the structure of separated material but only to show the chemical heterogeneity of some collected fractions. The  $^1\text{H}$  NMR spectra of crude and fractionated fractions of enoxaparin were significantly different (Fig. 7). The signals of the anomeric protons located between 5.0 and 5.5 ppm, and those between 3.5 and 4.4 ppm show characteristic variations. The spectrum of fraction 72, corresponding to the first zone defined for the different criteria of analysis, is very different than the spectra of the fractions 77 and 83 which are quite similar. Nevertheless, they present some differences in the signals between 3.5 and 4.4 ppm.

## 4. Conclusion

We have shown that the CPC separation provides two main families of low-molecular-mass heparins as clearly seen by SEC and NMR analysis. The first

divides up in the first fractions (from 68 to 76). The detailed study of this zone is impossible because the second family is partially co-eluted. Indeed, the latter is eluted from fraction 68 to 87. So, except for HPSEC, the other analytical methods lead to averaged data in the first zone. From fraction 77 to 87 four sub-families are defined according to the different analytical methods (Table 2).

The anion-exchange displacement applied to centrifugal partition chromatography is efficient for the fractionation of low-molecular-mass heparins. Indeed, fractionation of polysulfated polysaccharides according to molecular mass and also sulfatation content using mildly basic conditions and without solid support is shown. Selectivity is induced by the stability of ion pairs that is in turn dependent on molecular mass, sulfur content and molecular geometry. As suggested by Chevolot et al. this procedure seems a good and simple method for fractionation of complex mixtures of anionic species that are water soluble at all pH values.

The displacement CPC using LA2 as a weak anion exchanger introduces a supplementary way of purifying complex mixtures, in combination with other preparative techniques such as gel permeation and ion-exchange on solid support. The introduction of a new selectivity criterion will be interesting in the framework of the biological activity evaluation of original heparin fractions.

## 5. Nomenclature

|                  |  |
|------------------|--|
| $e_0$            | in each cell: number of moles of Exchanger/ $V_{\text{aq}}$      |
| $K_e$            | autodissociation constant of water                               |
| $K_i$            | equilibrium constant for analyte $i$                             |
| $n$              | number of analytes   |
| $n_0$            | in each cell: number of moles of $\text{Na}^+$ / $V_{\text{aq}}$ |
| $s_i$            | in each cell: number of moles of analyte $i$ / $V_{\text{aq}}$   |
| $v$              | ratio $V_{\text{org}}/V_{\text{aq}}$                             |
| $V_{\text{aq}}$  | volume of the aqueous mobile phase in each cell                  |
| $V_{\text{org}}$ | volume of the organic stationary phase in each cell              |

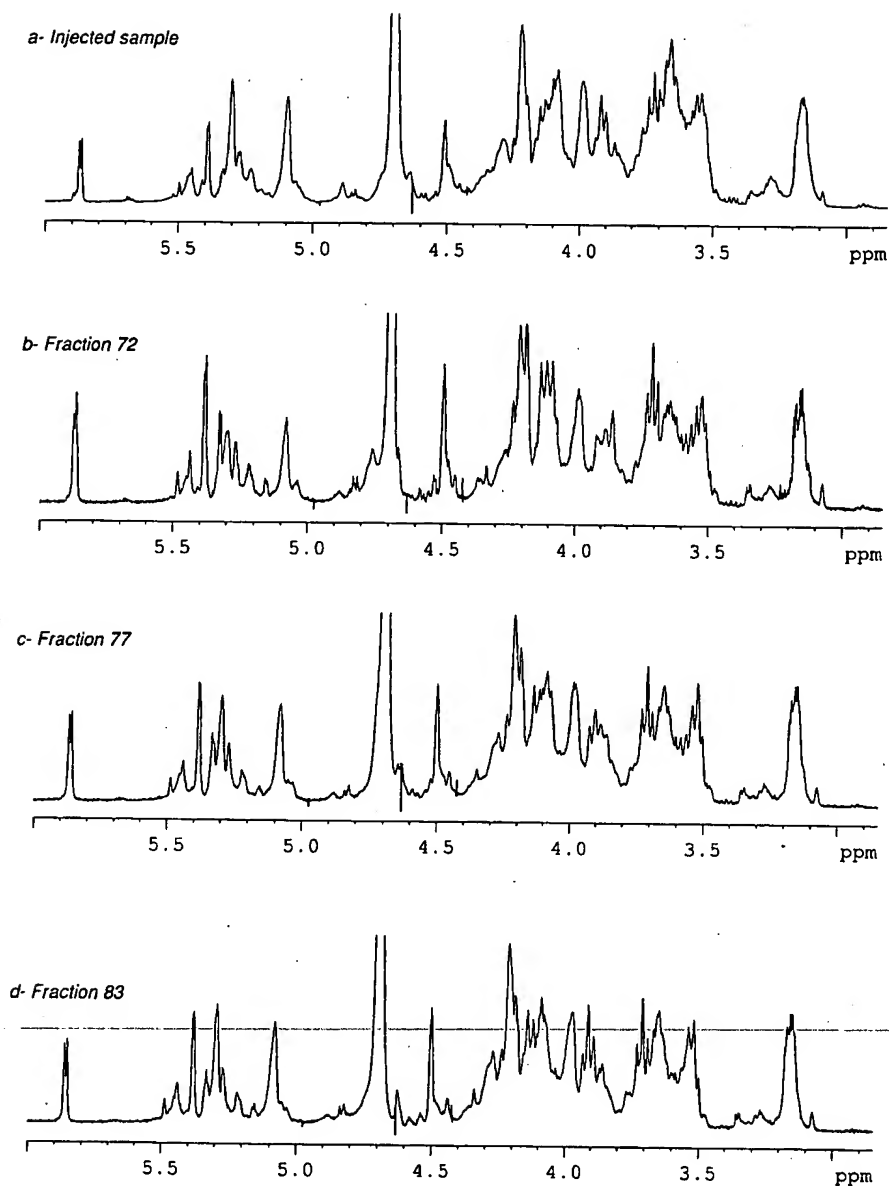


Fig. 7.  $^1\text{H}$  NMR spectra of three representative fractions and of the injected sample.

Table 2

Analytical characteristics of the separated families of low-molecular-mass heparins

| Zones (tubes) | Families | Retention time in the SEC analysis (min)                 | Average pH (SD) | Average sulphur content (SD) |
|---------------|----------|--|-----------------|------------------------------|
| [68–76]       | I+II     | $\text{Tr}_I$ : 12.4–12.15; $\text{Tr}_{II}$ : 14.8–13.8 | 6.38 (0.03)     | 10.17 (0.52)                 |
| [77–79]       | IIa      | $\text{Tr}_{IIa}$ : 14.8–13.8                            | 6.5 (0)         | 10.19 (0.02)                 |
| [80–81]       | IIb      | $\text{Tr}_{IIb}$ : 13.7                                 | 6.6 (0.05)      | 10.70 (0.40)                 |
| [82–83]       | IIc      | $\text{Tr}_{IIc}$ : 13.6                                 | 6.725 (0.25)    | 10.13 (0.24)                 |
| [84–87]       | IId      | $\text{Tr}_{IId}$ : 13.4–13                              | 11.4 (0.45)     | 4.18 (2.52)                  |

*Greek* $\alpha$ 

in each cell: concentration of the exchanger in the organic phase/concentration of  $\text{OH}^-$  in the aqueous phase

*Subscript* $i$ 

analyte number;  $i=0$  refers to  $\text{Cl}^-$

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